

1 **CYTOTOXICITY MEDIATION OF CELLS EVIDENCING SURFACE**

2 **EXPRESSION OF CD44**

3
4 **Reference to Related Applications:**

5 This application is a continuation-in-part of application S.N. 10/647,818 filed
6 August 22, 2003, which is a continuation-in-part of application S.N. 10/603,000, filed June
7 23, 2003, which is a continuation-in-part of application S.N. 09/727,361, filed November
8 29, 2000, which is a continuation-in-part of application S.N. 09/415,278, filed October 8,
9 1999, now U.S. Patent 6,180,357 B1, the contents of each of which are herein incorporated
10 by reference.

11 **Field Of The Invention:**

12 This invention relates to the diagnosis and treatment of cancerous diseases,
13 particularly to the mediation of cytotoxicity of tumor cells; and most particularly to the use
14 of cancerous disease modifying antibodies (CDMAB), optionally in combination with one
15 or more chemotherapeutic agents, as a means for initiating the cytotoxic response. The
16 invention further relates to binding assays, which utilize the CDMAB of the instant
17 invention.

18 **Background Of The Invention:**

19 Raising monoclonal antibodies against human white blood cells led to the
20 discovery of the CD44 antigen; a single chain hyaluronic acid (HA) binding glycoprotein
21 expressed on a wide variety of normal tissue and on all types of hematopoietic cells. It
22 was originally associated with lymphocyte activation and homing. Currently, its putative
23 physiological role also includes activation of inflammatory genes, modulation of cell cycle,

1 induction of cell proliferation, induction of differentiation and development, induction of
2 cytoskeletal reorganization and cell migration and cell survival/resistance to apoptosis.

3 In humans, the single gene copy of CD44 is located on the short arm of
4 chromosome 11, 11p13. The gene contains 19 exons; the first 5 are constant, the next 9
5 are variant, the following 3 are constant and the final 2 are variant. Differential splicing
6 can lead to over 1000 different isoforms. However, currently only several dozen naturally
7 occurring variants have been identified.

8 The CD44 standard glycoprotein consists of a N-terminal extracellular (including a
9 20 a.a. leader sequence, and a membrane proximal region (85 a.a.)) domain (270 a.a.), a
10 transmembrane region (21 a.a.) and a cytoplasmic tail (72 a.a.). The extracellular region
11 also contains a link module at the N-terminus. This region is 92 a.a. in length and shows
12 homology to other HA binding link proteins. There is high homology between the mouse
13 and human forms of CD44. The variant forms of the protein are inserted to the carboxy
14 terminus of exon 5 and are located extracellularly when expressed.

15 A serum soluble form of CD44 also occurs naturally and can arise from either a
16 stop codon (within the variable region) or from proteolytic activity. Activation of cells
17 from a variety of stimuli including TNF- α results in shedding of the CD44 receptor.
18 Shedding of the receptor has also been seen with tumor cells and can result in an increase
19 in the human serum concentration of CD44 by up to 10-fold. High CD44 serum
20 concentration suggests malignancy (ovarian cancer being the exception).

21 The standard form of CD44 exists with a molecular weight of approximately 37
22 kD. Post-translational modifications increase the molecular weight to 80-90 kD. These
23 modifications include amino terminus extracellular domain N-linked glycosylations at
24 asparagine residues, O-linked glycosylations at serine/threonine residues at the carboxy

1 terminus of the extracellular domain and glycosaminoglycan additions. Splice variants can
2 range in size from 80-250 kD.

3 HA, a polysaccharide located on the extracellular matrix (ECM) in mammals, is
4 thought to be the primary CD44 ligand. However, CD44 has also been found to bind such
5 proteins as collagen, fibronectin, laminin etc. There appears to be a correlation between
6 HA binding and glycosylation. Inactive CD44 (does not bind HA) has the highest levels of
7 glycosylation, active CD44 (binding HA) the lowest while inducible CD44 (does not or
8 weakly binds HA unless activated by cytokines, monoclonal antibodies, growth factors,
9 etc.) has glycosylation levels somewhere in between the active and inactive forms.

10 CD44 can mediate some of its functions through signal transduction pathways that
11 depend on the interaction of the cell, stimulus and the environment. Some of these
12 pathways include the NF κ B signaling cascade (involved in the inflammatory response), the
13 Ras-MAPK signal transduction pathway (involved with activating cell cycling and
14 proliferation), the Rho family of proteins (involved with cytoskeleton reorganization and
15 cell migration) and the PI3-K-related signaling pathway (related to cell survival). All of
16 the above-mentioned functions are closely associated with tumor disease initiation and
17 progression. CD44 has also been implicated in playing a role in cancer through a variety of
18 additional mechanisms. These include the presentation of growth factors, chemokines and
19 cytokines by cell surface proteoglycans present on the cell surface of CD44 to receptors
20 involved in malignancy. Also, the intracellular degradation of HA by lysosomal
21 hylauronidases after internalization of the CD44-HA complex can potentially increase the
22 likelihood of tumor invasiveness and induction of angiogenesis through the ECM. In
23 addition, the transmission of survival or apoptotic signals has been shown to occur through
24 either the standard or variable CD44 receptor. CD44 has also been suggested to be

1 involved in cell differentiation and migration. Many, if not all, of these mechanisms are
2 environment and cell dependent and several give rise to variable findings. Therefore, more
3 research is required before any conclusions can be drawn.

4 In order to validate a potential functional role of CD44 in cancer, expression
5 studies of CD44 were undertaken to determine if differential expression of the receptor
6 correlates with disease progression. However, inconsistent findings were observed in a
7 majority of tumor types and this is probably due to a combination of reagents, technique,
8 pathological scoring and cell type differences between researchers. Renal cell carcinoma
9 and non-Hodgkin's lymphoma appear to be the exception in that patients with high CD44
10 expressing tumors consistently had shorter survival times than their low or non-CD44
11 expressing counterparts.

12 Due to its association with cancer, CD44 has been the target of the development of
13 anti-cancer therapeutics. There is still controversy as to whether the standard or the variant
14 forms of CD44 are required for tumor progression. There is *in vivo* animal data to support
15 both views and again it may be tumor type and even cell type dependent. Different
16 therapeutic approaches have included injection of soluble CD44 proteins, hyaluronan
17 synthase cDNA, hyaluronidase, the use of CD44 antisense and CD44 specific antibodies.
18 Each approach has led to some degree of success thereby providing support for anti-CD44
19 cancer therapeutics.

20 Both variant and standard CD44 specific monoclonal antibodies have been
21 generated experimentally but for the most part these antibodies have no intrinsic biological
22 activity, rather they bind specifically to the type of CD44 they recognize. However, there
23 are some that are either active *in vitro* or *in vivo* but generally not both. Several anti-CD44
24 antibodies have been shown to mediate cellular events. For example the murine antibody

1 A3D8, directed against human erythrocyte Lutheran antigen CD44 standard form, was
2 shown to enhance CD2 (9-1 antibody) and CD3 (OKT3 antibody) mediated T cell
3 activation; another anti-CD44 antibody had similar effects. A3D8 also induced IL-1 release
4 from monocytes and IL-2 release from T lymphocytes. Interestingly, the use of A3D8 in
5 conjunction with drugs such as daunorubicin, mitoxantrone and etoposide inhibited
6 apoptosis induction in HL60 and NB4 AML cells by abrogating the generation of the
7 second messenger ceramide. The J173 antibody, which does not have intrinsic activity and
8 is directed against a similar epitope of CD44s, did not inhibit drug-induced apoptosis. The
9 NIH44-1 antibody, directed against an 85-110 kD and 200 kD form of CD44, augmented
10 T-cell proliferation through a pathway the authors speculated as either cross-linking or
11 aggregation of CD44. Taken together, there is no evidence that antibodies such as these are
12 suitable for use as cancer therapeutics since they either are not directed against cancer (e.g.
13 activate lymphocytes), induce cell proliferation, or when used with cytotoxic agents
14 inhibited drug-induced death of cancer cells.

15 Several anti-CD44 antibodies have been described which demonstrate anti-tumor
16 effects *in vivo*. The antibody 1.1ASML, a mouse IgG1 directed to the v6 variant of CD44,
17 has been shown to decrease the lymph node and lung metastases of the rat pancreatic
18 adenocarcinoma BSp73ASML. Survival of the treated animals was concomitantly
19 increased. The antibody was only effective if administered before lymph node
20 colonization, and was postulated to interfere with cell proliferation in the lymph node.
21 There was no direct cytotoxicity of the antibody on the tumor cells *in vitro*, and the
22 antibody did not enhance complement-mediated cytotoxicity, or immune effector cell
23 function. Utility of the antibody against human cells was not described.

1 Breyer *et al.* described the use of a commercially-available antibody to CD44s to
2 disrupt the progression of an orthotopically-implanted rat glioblastoma. The rat
3 glioblastoma cell line C6 was implanted in the frontal lobe, and after 1 week, the rats were
4 given 3 treatments with antibody by intracerebral injection. Treated rats demonstrated
5 decreased tumor growth, and higher body weight than buffer or isotype control treated rats.
6 The antibody was able to inhibit adhesion of cells *in vitro* to coverslips coated with
7 extracellular matrix components, but did not have any direct cytotoxic effects on cells.
8 This antibody was not tested against human cells.

9 A study was carried out which compared the efficacy of an antibody to CD44s (IM-
10 7.8.1) to an antibody to CD44v10 (K926). The highly metastatic murine melanoma line
11 B16F10, which expresses both CD44 isoforms, was implanted intravenously into mice.
12 After 2 days, antibodies were given every third day for the duration of the study. Both
13 antibodies caused a significant reduction of greater than 50% in the number of lung
14 metastases; there was no significant difference in efficacy between the two antibodies. The
15 antibody did not affect proliferation *in vitro*, and the authors, Zawadzki *et al.*, speculated
16 that the inhibition of tumor growth was due to the antibody blocking the interaction of
17 CD44 with its ligand. In another study using IM-7.8.1, Zahalka *et al.* demonstrated that
18 the antibody and its F(ab')₂ fragment were able to block the lymph node infiltration by the
19 murine T-cell lymphoma LB. This conferred a significant survival benefit to the mice.
20 Wallach-Dayana *et al.* showed that transfection of LB-TRs murine lymphoma, which does
21 not spontaneously form tumors, with CD44v4-v10 conferred the ability to form tumors.
22 IM-7.8.1 administration decreased tumor size of the implanted transfected cells in
23 comparison to the isotype control antibody. None of these studies demonstrated human
24 utility for this antibody.

1 GKW.A3, a mouse IgG2a, is specific for human CD44 and prevents the formation
2 and metastases of a human melanoma xenograft in SCID mice. The antibody was mixed
3 with the metastatic human cell line SMMU-2, and then injected subcutaneously.
4 Treatments were continued for the following 3 weeks. After 4 weeks, only 1 of 10 mice
5 developed a tumor at the injection site, compared to 100 percent of untreated animals.
6 F(ab')₂ fragments of the antibody demonstrated the same inhibition of tumor formation,
7 suggesting that the mechanism of action was not dependent on complement or antibody-
8 dependent cellular cytotoxicity. If the tumor cells were injected one week prior to the first
9 antibody injection, 80 percent of the animals developed tumors at the primary site.
10 However, it was noted that the survival time was still significantly increased. Although the
11 delayed antibody administration had no effect on the primary tumor formation, it
12 completely prevented the metastases to the lung, kidney, adrenal gland, liver and
13 peritoneum that were present in the untreated animals. This antibody does not have any
14 direct cytotoxicity on the cell line *in vitro* or does it interfere with proliferation of SMMU-
15 2 cells, and appears to have its major effect on tumor formation by affecting metastasis or
16 growth. One notable feature of this antibody was that it recognized all isoforms of CD44,
17 which suggests limited possibilities for therapeutic use.

18 Strobel *et al.* describe the use of an anti-CD44 antibody (clone 515) to inhibit the
19 peritoneal implantation of human ovarian cancer cells in a mouse xenograft model. The
20 human ovarian cell line 36M2 was implanted intraperitoneally into mice in the presence of
21 the anti-CD44 antibody or control antibody, and then treatments were administered over
22 the next 20 days. After 5 weeks, there were significantly fewer nodules in the peritoneal
23 cavity in the antibody treated group. The nodules from both the anti-CD44 and control
24 treated groups were the same size, suggesting that once the cells had implanted, the

1 antibody had no effect on tumor growth. When cells were implanted subcutaneously, there
2 was also no effect on tumor growth, indicating that the antibody itself did not have an anti-
3 proliferative or cytotoxic effect. In addition, there was no effect of the antibody on cell
4 growth *in vitro*.

5 VFF-18, also designated as BIWA 1, is a high-affinity antibody to the v6 variant of
6 CD44 specific for the 360-370 region of the polypeptide. This antibody has been used as a
7 ^{99m}Techetium-labelled conjugate in a Phase 1 clinical trial in 12 patients. The antibody
8 was tested for safety and targeting potential in patients with squamous cell carcinoma of
9 the head and neck. Forty hours after injection, 14 percent of the injected dose was taken
10 up by the tumor, with minimal accumulation in other organs including the kidney, spleen
11 and bone marrow. The highly selective tumor binding suggests a role for this antibody in
12 radioimmunotherapy, although the exceptionally high affinity of this antibody prevented
13 penetration into the deeper layers of the tumor. Further limiting the application of BIWA 1
14 is the immunogenicity of the murine antibody (11 of 12 patients developed human anti-
15 mouse antibodies (HAMA)), heterogenous accumulation throughout the tumor and
16 formation of antibody-soluble CD44 complexes. WO 02/094879 discloses a humanized
17 version of VFF-18 designed to overcome the HAMA response, designated BIWA 4.
18 BIWA 4 was found to have a significantly lower antigen binding affinity than the parent
19 VFF 18 antibody. Surprisingly, the lower affinity BIWA 4 antibody had superior tumor
20 uptake characteristics than the higher affinity BIWA 8 humanized VFF-18 antibody. Both
21 ^{99m}Techetium-labelled and ¹⁸⁶Rhenium-labelled BIWA 4 antibodies were assessed in a 33
22 patient Phase 1 clinical trial to determine safety, tolerability, tumor accumulation and
23 maximum tolerated dose, in the case of ¹⁸⁶Re-labelled BIWA 4. There appeared to be
24 tumor related uptake of ^{99m}Tc-labelled BIWA 4. There were no tumor responses seen with

1 all doses of ^{186}Re -labelled BIWA 4, although a number had stable disease; the dose
2 limiting toxicity occurred at 60 mCi/m². There was a 50-65 percent rate of adverse events
3 with 12 of 33 patients deemed to have serious adverse events (thrombocytopenia,
4 leucopenia and fever) and of those 6, all treated with ^{186}Re -labelled BIWA 4, died in the
5 course of treatment or follow-up due to disease progression. Two patients developed
6 human anti-human antibodies (HAHA). A Phase 1 dose escalation trial of ^{186}Re -labelled
7 BIWA 4 was carried out in 20 patients. Oral mucositis and dose-limiting thrombocytopenia
8 and leucocytopenia were observed; one patient developed a HAHA response. Stable
9 disease was seen in 5 patients treated at the highest dose of 60 mCi/m². Although deemed
10 to be acceptable in both safety and tolerability for the efficacy achieved, these studies
11 have higher rates of adverse events compared to other non-radioisotope conjugated
12 biological therapies in clinical studies. U.S. Patent Application US 2003/0103985 discloses
13 a humanized version of VFF-18 conjugated to a maytansinoid, designated BIWI 1, for use
14 in tumor therapy. A humanized VFF 18 antibody, BIWA 4, when conjugated to a toxin, i.e.
15 BIWI 1, was found to have significant anti-tumor effects in mouse models of human
16 epidermoid carcinoma of the vulva, squamous cell carcinoma of the pharynx or breast
17 carcinoma. The unconjugated version, BIWA 4, did not have anti-tumor effects and the
18 conjugated version, BIWI 1, has no evidence of safety or efficacy in humans.

19 Mab U36 is a murine monoclonal IgG1 antibody generated by UM-SCC-22B
20 human hypopharyngeal carcinoma cell immunization and selection for cancer and tissue
21 specificity. Antigen characterization through cDNA cloning and sequence analysis
22 identified the v6 domain of keratinocyte-specific CD44 splice variant epican as the target
23 of Mab U36. Immunohistochemistry studies show the epitope to be restricted to the cell
24 membrane. Furthermore, Mab U36 labeled 94 percent of the head and neck squamous cell

1 carcinomas (HNSCC) strongly, and within these tumors there was uniformity in cell
2 staining. A 10 patient ^{99m}Tc -labelled Mab U36 study showed selective accumulation of the
3 antibody to HNSCC cancers (20.4 +/- 12.4 percent injected dose/kg at 2 days); no adverse
4 effects were reported but two patients developed HAMA. In a study of radio-iodinated
5 murine Mab U36 there were 3 cases of HAMA in 18 patients and selective homogenous
6 uptake in HNSCC. In order to decrease the antigenicity of Mab U36 and decrease the rate
7 of HAMA a chimeric antibody was constructed. Neither the chimeric nor the original
8 murine Mab U36 has ADCC activity. There is no evidence of native functional activity of
9 Mab U36. ^{186}Re -labelled chimeric Mab U36 was used to determine the utility of Mab U36
10 as a therapeutic agent. In this Phase 1 escalating dose trial 13 patients received a scouting
11 dose of ^{99m}Tc -labelled chimeric Mab U36 followed by ^{186}Re -labelled chimeric Mab U36.
12 There were no acute adverse events reported but following treatment dose limiting
13 myelotoxicity (1.5 GBq/m^2) in 2 of 3 patients, and thrombocytopenia in one patient treated
14 with the maximum tolerated dose (1.0 GBq/m^2) were observed. Although there were some
15 effects on tumor size these effects did not fulfill the criteria for objective responses to
16 treatment. A further study of ^{186}Re -labelled chimeric Mab U36 employed a strategy of
17 using granulocyte colony-stimulating factor stimulated whole blood reinfusion to double
18 the maximum-tolerated activity to 2.8 Gy. In this study of nine patients with various
19 tumors of the head and neck 3 required transfusions for drug related anemia. Other toxicity
20 includes grade 3 myelotoxicity, and grade 2 mucositis. No objective tumor responses were
21 reported although stable disease was achieved for 3-5 months in 5 patients. Thus, it can be
22 seen that although Mab U36 is a highly specific antibody the disadvantage of requiring a
23 radioimmunoconjugate to achieve anti-cancer effects limits its usefulness because of the
24 toxicity associated with the therapy in relation to the clinical effects achieved.

1 To summarize, a CD44v6 (1.1ASML) and CD44v10 (K926) monoclonal antibody
2 have been shown to reduce metastatic activity in rats injected with a metastatic pancreatic
3 adenocarcinoma or mice injected with a malignant melanoma respectively. Another anti-
4 CD44v6 antibody (VFF-18 and its derivatives), only when conjugated to a maytansinoid or
5 a radioisotope, has been shown to have anti-tumor effects. Anti-standard CD44
6 monoclonal antibodies have also been shown to suppress intracerebral progression by rat
7 glioblastoma (anti-CD44s), lymph node invasion by mouse T cell lymphoma (IM-7.8.1) as
8 well as inhibit implantation of a human ovarian cancer cell line in nude mice (clone 515),
9 lung metastasis of a mouse melanoma cell line (IM-7.8.1) and metastasis of a human
10 melanoma cell line in SCID mice (GKW.A3). The radioisotope conjugated Mab U36 anti-
11 CD44v6 antibody and its derivatives had anti-tumor activity in clinical trials that were
12 accompanied by significant toxicity. These results, though they are encouraging and
13 support the development of anti-CD44 monoclonal antibodies as potential cancer
14 therapeutics, demonstrate limited effectiveness, safety, or applicability to human cancers.

15 Thus, if an antibody composition were isolated which mediated cancerous cell
16 cytotoxicity, as a function of its attraction to cell surface expression of CD44 on said cells,
17 a valuable diagnostic and therapeutic procedure would be realized.

18

19 Prior Patents:

20 U.S. Patent No. 5,750,102 discloses a process wherein cells from a patient's tumor
21 are transfected with MHC genes, which may be cloned from cells or tissue from the
22 patient. These transfected cells are then used to vaccinate the patient.

1 U.S. Patent No. 4,861,581 discloses a process comprising the steps of obtaining
2 monoclonal antibodies that are specific to an internal cellular component of neoplastic and
3 normal cells of the mammal but not to external components, labeling the monoclonal
4 antibody, contacting the labeled antibody with tissue of a mammal that has received
5 therapy to kill neoplastic cells, and determining the effectiveness of therapy by measuring
6 the binding of the labeled antibody to the internal cellular component of the degenerating
7 neoplastic cells. In preparing antibodies directed to human intracellular antigens, the
8 patentee recognizes that malignant cells represent a convenient source of such antigens.

9 U.S. Patent No. 5,171,665 provides a novel antibody and method for its production.
10 Specifically, the patent teaches formation of a monoclonal antibody which has the property
11 of binding strongly to a protein antigen associated with human tumors, e.g. those of the
12 colon and lung, while binding to normal cells to a much lesser degree.

13 U.S. Patent No. 5,484,596 provides a method of cancer therapy comprising
14 surgically removing tumor tissue from a human cancer patient, treating the tumor tissue to
15 obtain tumor cells, irradiating the tumor cells to be viable but non-tumorigenic, and using
16 these cells to prepare a vaccine for the patient capable of inhibiting recurrence of the
17 primary tumor while simultaneously inhibiting metastases. The patent teaches the
18 development of monoclonal antibodies, which are reactive with surface antigens of tumor
19 cells. As set forth at col. 4, lines 45 et seq., the patentees utilize autochthonous tumor cells
20 in the development of monoclonal antibodies expressing active specific immunotherapy in
21 human neoplasia.

22 U.S. Patent No. 5,693,763 teaches a glycoprotein antigen characteristic of human
23 carcinomas and not dependent upon the epithelial tissue of origin.

1 U.S. Patent No. 5,783,186 is drawn to anti-Her2 antibodies, which induce apoptosis
2 in Her2 expressing cells, hybridoma cell lines producing the antibodies, methods of
3 treating cancer using the antibodies and pharmaceutical compositions including said
4 antibodies.

5 U.S. Patent No. 5,849,876 describes new hybridoma cell lines for the production of
6 monoclonal antibodies to mucin antigens purified from tumor and non-tumor tissue
7 sources.

8 U.S. Patent No. 5,869,268 is drawn to a method for generating a human
9 lymphocyte producing an antibody specific to a desired antigen, a method for producing a
10 monoclonal antibody, as well as monoclonal antibodies produced by the method. The
11 patent is particularly drawn to the production of an anti-HD human monoclonal antibody
12 useful for the diagnosis and treatment of cancers.

13 U.S. Patent No. 5,869,045 relates to antibodies, antibody fragments, antibody
14 conjugates and single chain immunotoxins reactive with human carcinoma cells. The
15 mechanism by which these antibodies function is 2-fold, in that the molecules are reactive
16 with cell membrane antigens present on the surface of human carcinomas, and further in
17 that the antibodies have the ability to internalize within the carcinoma cells, subsequent to
18 binding, making them especially useful for forming antibody-drug and antibody-toxin
19 conjugates. In their unmodified form the antibodies also manifest cytotoxic properties at
20 specific concentrations.

21 U.S. Patent No. 5,780,033 discloses the use of autoantibodies for tumor therapy and
22 prophylaxis. However, this antibody is an anti-nuclear autoantibody from an aged
23 mammal. In this case, the autoantibody is said to be one type of natural antibody found in

1 the immune system. Because the autoantibody comes from "an aged mammal", there is no
2 requirement that the autoantibody actually comes from the patient being treated. In
3 addition the patent discloses natural and monoclonal antinuclear autoantibody from an
4 aged mammal, and a hybridoma cell line producing a monoclonal antinuclear
5 autoantibody.

6 U.S. Patent No. 5,916,561 discloses a specific antibody, VFF-18, and its variants
7 directed against the variant exon v6 of the CD44 gene. This antibody is an improvement
8 over the comparator antibody in that it recognizes a human CD44 v6 variant rather than a
9 rat CD44 v6 variant. In addition this antibody discloses diagnostic assays for CD44 v6
10 expression. There was no *in vitro* or *in vivo* function disclosed for this antibody.

11 U.S. Patent No. 5,616,468 discloses a monoclonal antibody, Var3.1, raised against
12 a synthetic peptide containing a sequence encoded by the human exon 6A of the CD44
13 gene. Specifically this antibody does not bind to the 90 kD form of human CD44 and is
14 distinguished from the Hermes-3 antibody. A method for detection of the v6 variant of
15 CD44 is provided, as well as a method for screening and assaying for malignant
16 transformation based on this antigen. A method for screening for inflammatory disease
17 based on detecting the antigen in serum is also provided.

18 U.S. Patent No. 5,879,898 discloses a specific antibody that binds to a 129 bp exon
19 of a human CD44 variant 6 that produces a 43 amino acid peptide. The monoclonal
20 antibody is produced by a number of hybridoma cell lines: MAK<CD44>M-1.1.12,
21 MAK<CD44>M-2.42.3, MAK<CD44>M-4.3.16. The antibody is generated from a fusion
22 protein that contains at least a hexapeptide of the novel CD44 v6 amino acid sequence.
23 Further, there is a disclosure of an immunoassay for the detection of exon 6 variant that

1 can be used as a cancer diagnostic. Significantly, there is no *in vitro* or *in vivo* function of
2 this antibody disclosed.

3 U.S. Patent No. 5,942,417 discloses a polynucleotide that encodes a CD44 like
4 polypeptide, and the method of making a recombinant protein using the polynucleotide and
5 its variants. Antibodies are claimed to these polypeptides however there are no specific
6 examples and there are no deposited clones secreting such antibodies. Northern blots
7 demonstrate the appearance of the polynucleotide in several types of tissues, but there is no
8 accompanying evidence that there is translation and expression of this polynucleotide.
9 Therefore, there is no evidence that there were antibodies to be made to the gene product
10 of this polynucleotide, that these antibodies would have either *in vitro* or *in vivo* function,
11 and whether they would be relevant to human cancerous disease.

12 U.S. Patent No. 5,885,575 discloses an antibody that reacts with a variant epitope
13 of CD44 and methods of identifying the variant through the use of the antibody. The
14 isolated polynucleotide encoding this variant was isolated from rat cells, and the antibody,
15 mAb1.1ASML, directed against this variant recognizes proteins of molecular weight 120
16 kD, 150 kD, 180 kD, and 200 kD. The administration of monoclonal antibody 1.1ASML
17 delayed the growth and metastases of rat BSp73ASML in isogenic rats. Significantly
18 1.1ASML does not recognize human tumors as demonstrated by its lack of reactivity to
19 LCLC97 human large-cell lung carcinoma. A human homolog was isolated from LCLC97
20 but no equivalent antibody recognizing this homolog was produced. Thus, although an
21 antibody specific to a variant of rat CD44 was produced and shown to affect the growth
22 and metastasis of rat tumors there is no evidence for the effect the this antibody against
23 human tumors. More specifically the inventors point out that this antibody does not
24 recognize human cancers.

1 Summary Of The Invention:

2 The instant inventors have previously been awarded U.S. Patent 6,180,357, entitled
3 "Individualized Patient Specific Anti-Cancer Antibodies" directed to a process for
4 selecting individually customized anti-cancer antibodies, which are useful in treating a
5 cancerous disease. For the purpose of this document, the terms "antibody" and
6 "monoclonal antibody" (mAb) may be used interchangeably and refer to intact
7 immunoglobulins produced by hybridomas (e.g. murine or human), immunoconjugates
8 and, as appropriate, immunoglobulin fragments and recombinant proteins derived from
9 said immunoglobulins, such as chimeric and humanized immunoglobulins, F(ab') and
10 F(ab')₂ fragments, single-chain antibodies, recombinant immunoglobulin variable regions
11 (Fv)s, fusion proteins etc. It is well recognized in the art that some amino acid sequence
12 can be varied in a polypeptide without significant effect on the structure or function of the
13 protein. In the molecular rearrangement of antibodies, modifications in the nucleic or
14 amino acid sequence of the backbone region can generally be tolerated. These include, but
15 are not limited to, substitutions (preferred are conservative substitutions), deletions or
16 additions. Furthermore, it is within the purview of this invention to conjugate standard
17 chemotherapeutic modalities, e.g. radionuclides, with the CDMAB of the instant invention,
18 thereby focusing the use of said chemotherapeutics. The CDMAB can also be conjugated
19 to toxins, cytotoxic moieties, enzymes e.g. biotin conjugated enzymes, or hematogenous
20 cells, whereby an antibody conjugate is formed.

21 This application utilizes substantially the method for producing patient specific
22 anti-cancer antibodies as taught in the '357 patent for isolating hybridoma cell lines which
23 encode for cancerous disease modifying monoclonal antibodies. These antibodies can be
24 made specifically for one tumor and thus make possible the customization of cancer

1 therapy. Within the context of this application, anti-cancer antibodies having either cell-
2 killing (cytotoxic) or cell-growth inhibiting (cytostatic) properties will hereafter be referred
3 to as cytotoxic. These antibodies can be used in aid of staging and diagnosis of a cancer,
4 and can be used to treat tumor metastases as well as primary tumors.

5 The prospect of individualized anti-cancer treatment will bring about a change in
6 the way a patient is managed. A likely clinical scenario is that a tumor sample is obtained
7 at the time of presentation, and banked. From this sample, the tumor can be typed from a
8 panel of pre-existing cancerous disease modifying antibodies. The patient will be
9 conventionally staged but the available antibodies can be of use in further staging the
10 patient. The patient can be treated immediately with the existing antibodies and/or a panel
11 of antibodies specific to the tumor can be produced either using the methods outlined
12 herein or through the use of phage display libraries in conjunction with the screening
13 methods herein disclosed. All the antibodies generated will be added to the library of anti-
14 cancer antibodies since there is a possibility that other tumors can bear some of the same
15 epitopes as the one that is being treated. The antibodies produced according to this method
16 may be useful to treat cancerous disease in any number of patients who have cancers that
17 bind to these antibodies.

18 Using substantially the process of US 6,180,357, the mouse monoclonal antibody
19 H460-16-2 was obtained following immunization of mice with cells from a patient's lung
20 tumor biopsy. The H460-16-2 antigen was expressed on the cell surface of a broad range
21 of human cell lines from different tissue origins. The breast cancer cell line MDA-MB-
22 231 (MB-231) and skin cancer cell line A2058 were susceptible to the cytotoxic effects of
23 H460-16-2 *in vitro*.

1 The result of H460-16-2 cytotoxicity against MB-231 cells in culture was further
2 extended by its anti-tumor activity towards these cancer cells when transplanted into mice
3 (as disclosed in S.N. 10/603,000). Pre-clinical xenograft tumor models are considered
4 valid predictors of therapeutic efficacy.

5 In the preventative *in vivo* model of human breast cancer, H460-16-2 treatment was
6 significantly ($p<0.0001$) more effective in suppressing tumor growth during the treatment
7 period than an isotype control antibody, which was identical to H460-16-2 in structure and
8 size but incapable of binding MB-231 cells. At the end of the treatment phase, mice given
9 H460-16-2 had tumors that grew to only 1.3 percent of the control group. During the post
10 treatment follow-up period, the treatment effects of H460-16-2 were sustained and the
11 mean tumor volume in the treated groups continued to be significantly smaller than
12 controls until the end of the measurement phase. Using survival as a measure of antibody
13 efficacy, it was estimated that the risk of dying in the H460-16-2 treatment group was
14 about 71 percent of the antibody buffer control group ($p=0.028$) at 70 days post-treatment.
15 These data demonstrated that H40-16-2 treatment conferred a survival benefit compared to
16 the control-treated groups. H460-16-2 treatment appeared safe, as it did not induce any
17 signs of toxicity, including reduced body weight and clinical distress. Thus, H460-16-2
18 treatment was efficacious as it both delayed tumor growth and enhanced survival compared
19 to the control-treated groups in a well-established model of human breast cancer.

20 In addition, H460-16-2 demonstrated anti-tumor activity against MB-231 cells in
21 an established *in vivo* tumor model (as outlined in S.N. 10/603,000). Treatment with H460-
22 16-2 was compared to the standard chemotherapeutic drug, Cisplatin, and it was shown
23 that the Cisplatin and H460-16-2 treatment groups had significantly ($p<0.001$) smaller
24 mean tumor volumes compared with groups treated with either antibody dilution buffer or

1 the isotype control antibody. H460-16-2 treatment mediated tumor suppression that was
2 approximately two-thirds that of cisplatin chemotherapy but without the significant (19.2
3 percent) weight loss ($p<0.003$) and clinical distress, including 2 treatment-associated
4 deaths, observed with Cisplatin treatment. The anti-tumor activity of H460-16-2 and its
5 minimal toxicity make it an attractive anti-cancer therapeutic agent. In the post-treatment
6 period, H460-16-2 showed a significant survival benefit ($p<0.02$) as the risk of dying in the
7 H460-16-2 group was about half of that in the isotype control antibody group at >70 days
8 after treatment. The observed survival benefit continued past 120 days post-treatment
9 where 100 percent of the isotype control and cisplatin treated mice had died compared to
10 67 percent of the H460-16-2 treatment group. H460-16-2 maintained tumor suppression
11 by delaying tumor growth by 26 percent compared to the isotype control antibody group.
12 At 31 days post treatment, H460-16-2 limited tumor size by reducing tumor growth by 48
13 percent compared to the isotype control group, which is comparable to the 49 percent
14 reduction observed at the end of the treatment. In the established tumor model of breast
15 cancer, these results indicated the potential of H460-16-2 to maintain tumor suppression
16 beyond the treatment phase and demonstrated the ability of the antibody to reduce the
17 tumor burden and enhance survival in a mammal.

18 In addition to the beneficial effects in the established *in vivo* tumor model of breast
19 cancer, H460-16-2 treatment in combination with a chemotherapeutic drug (Cisplatin) had
20 anti-tumor activity against PC-3 cells in an established *in vivo* prostate cancer model.
21 Using a paired t-test, H460-16-2 plus Cisplatin treatment was significantly more effective
22 in suppressing tumor growth shortly after the treatment period than buffer control
23 ($p<0.0001$), Cisplatin treatment alone ($p=0.004$) or H460-16-2 treatment alone ($p<0.0001$).
24 At the end of the treatment phase, mice given H460-16-2 plus Cisplatin had tumors that

1 grew to only 28.5 percent of the buffer control group. For PC-3 SCID xenograft models,
2 body weight can be used as a surrogate indicator of disease progression. Mice in all the
3 groups experienced severe weight loss. In this study, mice in all groups showed a weight
4 loss of approximately 23 to 35 percent by the end of the treatment period. The group
5 treated with H460-16-2 showed the smallest degree of weight loss (21.7 percent). After
6 treatment, day 48, there was no significant increase in weight loss associated with the
7 treatment of H460-16-2 and Cisplatin in comparison to buffer control ($p=0.5042$). Thus,
8 H460-16-2 plus Cisplatin treatment was efficacious as it delayed tumor growth compared
9 to the isotype control treated group in a well-established model of human prostate cancer.

10 In order to validate the H460-16-2 epitope as a drug target, the expression of
11 H460-16-2 antigen in normal human tissues was previously determined (S.N. 10/603,000).
12 This work was extended by comparison with the anti-CD44 antibodies; clone L178
13 (outlined in S.N. 10/647,818) and clone BU75 (outlined herein). By IHC staining with
14 H460-16-2, the majority of the tissues failed to express the H460-16-2 antigen, including
15 the cells of the vital organs, such as the liver, kidney (except for marginal staining of
16 tubular epithelial cells), heart, and lung. Results from tissue staining indicated that H460-
17 16-2 showed restricted binding to various cell types but had binding to infiltrating
18 macrophages, lymphocytes, and fibroblasts. The BU75 antibody showed a similar staining
19 pattern. However, there was at least one difference of note; staining of lymphocytes was
20 more intense with BU75 in comparison to H460-16-2.

21 Localization of the H460-16-2 antigen and determining its prevalence within the
22 population, such as among breast cancer patients, is important in assessing the therapeutic
23 use of H460-16-2 and designing effective clinical trials. To address H460-16-2 antigen
24 expression in breast tumors from cancer patients, tumor tissue samples from 50 individual

1 breast cancer patients were previously screened for expression of the H460-16-2 antigen
2 (S.N. 10/603,000) and was compared to L178 (S.N. 10/647,818). Current work compared
3 the staining of H460-16-2 to BU75 and the anti-Her2 antibody c-erbB-2. The results of the
4 current study were similar to previous results and showed that 62 percent of tissue samples
5 stained positive for the H460-16-2 antigen while 73 percent of breast tumor tissues were
6 positive for the BU75 epitope. Expression of H460-16-2 within patient samples appeared
7 specific for cancer cells as staining was restricted to malignant cells. H460-16-2 stained 4
8 of 10 samples of normal tissue from breast cancer patients while BU75 stained 8. Breast
9 tumor expression of both the H460-16-2 and BU75 antigen appeared to be mainly
10 localized to the cell membrane of malignant cells, making CD44 an attractive target for
11 therapy. H460-16-2 expression was further evaluated based on breast tumor expression of
12 the receptors for the hormones estrogen and progesterone, which play an important role in
13 the development, treatment, and prognosis of breast tumors. No correlation was apparent
14 between expression of the H460-16-2 antigen and expression of the receptors for either
15 estrogen or progesterone. When tumors were analyzed based on their stage, or degree to
16 which the cancer advanced, again there was no clear correlation between H460-16-2
17 antigen expression and tumor stage. Similar results were obtained with BU75. In
18 comparison to c-erbB-2, H460-16-2 showed a completely different staining profile where
19 52 percent of the breast tumor tissue samples that were positive for the H460-16-2 antigen
20 were negative for Her2 expression indicating a yet unmet targeted therapeutic need for
21 breast cancer patients. There were also differences in the intensity of staining between the
22 breast tumor tissue sections that were positive for both H460-16-2 and Her2. The c-erbB-2
23 antibody also positively stained one of the normal breast tissue sections.

1 To further extend the potential therapeutic benefit of H460-16-2, the frequency and
2 localization of the antigen within various human cancer tissues was also previously
3 determined (S.N. 10/603,000) and was compared to clone L178 (S.N. 10/647,818). The
4 majority of these tumor types were also positive for the L178 antigen. As with human
5 breast tumor tissue, H460-16-2 and L178 localization occurred on the membrane of tumor
6 cells. However, there was substantially more membrane localization with the L178
7 compared to the H460-16-2 antibody. Also, of the tumor types that were stained by both
8 H460-16-2 and L178, 43 percent of the tissues showed higher intensity staining with the
9 L178 antibody.

10 There appears to be no form of CD44 that exactly matches the IHC data presented
11 herein based on comparisons with the IHC data from the literature. The standard form of
12 CD44 is normally expressed in the human brain; H460-16-2 antigen is not. Antibodies
13 directed against pan-CD44 isoforms do not stain the liver (including Kupffer cells) and
14 positively stain the endometrial glands in all phases of the reproductive cycle. The H460-
15 16-2 antigen is clearly present on Kupffer cells and is only present on the secretory
16 endometrial glands of the reproductive cycle. H460-16-2 antigen is clearly present on
17 tissue macrophages and only the variant forms V4/5 and V8/9 show occasional
18 macrophage staining. The similar yet distinct binding pattern seen with H460-16-2 in
19 comparison to anti-CD44 L178 and now BU75 indicates that the H460-16-2 antigen is an
20 unique epitope of CD44.

21 As outlined previously (S.N. 10/647,818), additional biochemical data also
22 indicated that the antigen recognized by H460-16-2 is one of the forms of CD44. This was
23 supported by studies that showed a monoclonal antibody (L178) reactive against CD44
24 identifies proteins that were bound to H460-16-2 by immunoprecipitation. Western

1 blotting studies also suggested that the epitope of CD44 recognized by H460-16-2 was not
2 present on v6 or v10. The H460-16-2 epitope was also distinguished by being carbohydrate
3 and conformation dependent, whereas many anti-CD44 antibodies are directed against
4 peptide portions of CD44. These IHC and biochemical results demonstrated that H460-16-
5 2 binds to a variant of the CD44 antigen. Thus, the preponderance of evidence showed that
6 H460-16-2 mediates anti-cancer effects through ligation of an unique carbohydrate
7 dependent conformational epitope present on a variant of CD44. For the purpose of this
8 invention, said epitope is defined as a "CD44 antigenic moiety" characterized by its ability
9 to bind with a monoclonal antibody encoded by the hybridoma cell line H460-16-2,
10 antigenic binding fragments thereof or antibody conjugates thereof.

11 In order to further elucidate the mechanism behind H460-16-2's anti-cancer effects,
12 hyaluronic acid (HA) binding assays were performed. It was determined that an average
13 concentration of 1.87 (+/- 1.01) µg/mL of H460-16-2 was required to yield 50 percent
14 adhesion of MDA-MB-231 cells to HA. These results indicated that H460-16-2 interacts
15 with, at least in part, the region(s) on CD44 that are responsible for binding to HA and
16 consequently could be elucidating its anti-cancer effects through down regulation of
17 angiogenesis or tumor invasiveness through the ECM. In addition to the HA binding
18 assays, a cell cycling experiment was performed in order to determine if the H460-16-2 *in*
19 *vitro* and *in vivo* anti-cancer effects were due to regulation of the cell cycle. After 24 hrs
20 and with 20 µg/mL of H460-16-2, there was an increase in the number of MDA-MB-231
21 apoptotic cells in comparison to the isotype control. This effect also appeared to be dose
22 dependent. Therefore, the efficacy of H460-16-2 might be also due, in whole or in part, to
23 its apoptotic inducing capabilities.

24 *In toto*, this data demonstrates that the H460-16-2 antigen is a cancer associated
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1 antigen and is expressed in humans, and is a pathologically relevant cancer target. Further,
2 this data also demonstrates the binding of the H460-16-2 antibody to human cancer tissues,
3 and can be used appropriately for assays that can be diagnostic, predictive of therapy, or
4 prognostic. In addition, the cell membrane localization of this antigen is indicative of the
5 cancer status of the cell due to the lack of expression of the antigen in most non-malignant
6 cells, and this observation permits the use of this antigen, its gene or derivatives, its protein
7 or its variants to be used for assays that can be diagnostic, predictive of therapy, or
8 prognostic.

9 Other studies, involving the use of anti-CD44 antibodies, have limitations of
10 therapeutic potential that is not exhibited by H460-16-2. H460-16-2 demonstrates both *in*
11 *vitro* and *in vivo* anti-tumor activity. Previously described antibodies such
12 MAK<CD44>M-1.1.12, MAK<CD44>M-2.42.3 and MAK<CD44>M-4.3.16 have no *in*
13 *vitro* or *in vivo* cytotoxicity ascribed to them and VFF-18 and Mab U36 shows no intrinsic
14 tumor cytotoxicity. In addition other anti-CD44 antibodies that have shown *in vivo* tumor
15 effects also have certain limitations that are not evident with H460-16-2. For example,
16 ASML1.1, K926, anti-CD44s and IM-78.1 show *in vivo* anti-tumor activity against rat,
17 murine, rat and murine tumors grown in xenograft models respectively. H460-16-2
18 demonstrates anti-tumor activity in a model of human cancer. H460-16-2 is also directed
19 against human CD44 while antibodies such as ASML1.1 recognize only rat CD44. The
20 clone 515 anti-CD44 antibody does inhibit peritoneal tumor implantation of a human
21 ovarian cell line but does not prevent or inhibit tumor growth. H460-16-2 is capable of
22 inhibiting human breast tumor growth in a SCID mouse xenograft model. GKW.A3 is an
23 anti-human CD44 monoclonal antibody capable of inhibiting tumor growth of a human
24 metastasizing melanoma grown in mice in a preventative but not an established model.

1 H460-16-2 has demonstrated significant anti-tumor activity in both preventative and
2 established murine xenograft models of human breast cancer. Consequently, it is quite
3 apparent that H460-16-2 has superior anti-tumor properties in comparison to previously
4 described anti-CD44 antibodies. It has demonstrated both *in vitro* and *in vivo* anti-tumor
5 activity on a human breast tumor in SCID mice and is directed against human CD44. It
6 also exhibits activity in a preventative and established (more clinically relevant) model of
7 human breast cancer and it exhibits activity with Cisplatin in an established model of
8 human prostate cancer.

9 In all, this invention teaches the use of the H460-16-2 antigen as a target for a
10 therapeutic agent, that when administered can reduce the tumor burden of a cancer
11 expressing the antigen in a mammal (thus delaying disease progression), and can also lead
12 to a prolonged survival of the treated mammal. This invention also teaches the use of a
13 CDMAB (H460-16-2), and its derivatives, to target its antigen to reduce the tumor burden
14 of a cancer expressing the antigen in a mammal, and to prolong the survival of a mammal
15 bearing tumors that express this antigen. In addition, this invention teaches that after
16 binding to its antigen, H460-16-2 can interfere with a cancer cell's ability to interact with
17 hyaluronic acid and can also cause a cancer cell to undergo apoptosis. Furthermore, this
18 invention also teaches the use of detecting the H460-16-2 antigen in cancerous cells that
19 can be useful for the diagnosis, prediction of therapy, and prognosis of mammals bearing
20 tumors that express this antigen.

21 If a patient is refractory to the initial course of therapy or metastases develop, the
22 process of generating specific antibodies to the tumor can be repeated for re-treatment.
23 Furthermore, the anti-cancer antibodies can be conjugated to red blood cells obtained from

1 that patient and re-infused for treatment of metastases. There have been few effective
2 treatments for metastatic cancer and metastases usually portend a poor outcome resulting
3 in death. However, metastatic cancers are usually well vascularized and the delivery of
4 anti-cancer antibodies by red blood cells can have the effect of concentrating the antibodies
5 at the site of the tumor. Even prior to metastases, most cancer cells are dependent on the
6 host's blood supply for their survival and anti-cancer antibody conjugated to red blood
7 cells can be effective against *in situ* tumors as well. Alternatively, the antibodies may be
8 conjugated to other hematogenous cells, e.g. lymphocytes, macrophages, monocytes,
9 natural killer cells, etc.

10 There are five classes of antibodies and each is associated with a function that is
11 conferred by its heavy chain. It is generally thought that cancer cell killing by naked
12 antibodies are mediated either through antibody-dependent cell-mediated cytotoxicity
13 (ADCC) or complement-dependent cytotoxicity (CDC). For example murine IgM and
14 IgG2a antibodies can activate human complement by binding the C-1 component of the
15 complement system thereby activating the classical pathway of complement activation
16 which can lead to tumor lysis. For human antibodies, the most effective complement
17 activating antibodies are generally IgM and IgG1. Murine antibodies of the IgG2a and
18 IgG3 isotype are effective at recruiting cytotoxic cells that have Fc receptors which will
19 lead to cell killing by monocytes, macrophages, granulocytes and certain lymphocytes.
20 Human antibodies of both the IgG1 and IgG3 isotype mediate ADCC.

21 Another possible mechanism of antibody mediated cancer killing may be through
22 the use of antibodies that function to catalyze the hydrolysis of various chemical bonds in

1 the cell membrane and its associated glycoproteins or glycolipids, so-called catalytic
2 antibodies.

3 There are two additional mechanisms of antibody mediated cancer cell killing
4 which are more widely accepted. The first is the use of antibodies as a vaccine to induce
5 the body to produce an immune response against the putative antigen that resides on the
6 cancer cell. The second is the use of antibodies to target growth receptors and interfere
7 with their function or to down regulate that receptor so that effectively its function is lost.

8 Accordingly, it is an objective of the invention to utilize a method for producing
9 cancerous disease modifying antibodies from cells derived from a particular individual
10 which are cytotoxic with respect to cancer cells while simultaneously being relatively non-
11 toxic to non-cancerous cells, in order to isolate hybridoma cell lines and the corresponding
12 isolated monoclonal antibodies and antigen binding fragments thereof for which said
13 hybridoma cell lines are encoded.

14 It is an additional objective of the invention to teach methods of utilizing the
15 isolated monoclonal antibody or antigen binding fragment thereof encoded by the clone
16 deposited with the ATCC as PTA-4621 for determining a presence of cells which express a
17 CD44 antigenic moiety which specifically binds to an isolated monoclonal antibody or
18 antigen binding fragment thereof encoded by the clone deposited with the ATCC as PTA-
19 4621.

20 It is yet a further objective of the instant invention to teach methods for enhancing
21 the survival of a patient having a cancerous disease via the use of an isolated monoclonal
22 antibody or antigen binding fragment thereof encoded by the clone deposited with the
23 ATCC as PTA-4621, which antibody specifically binds to a CD44 antigenic moiety.

1 It is an additional objective of the invention to teach CDMAB and antigen binding
2 fragments thereof.

3 It is a further objective of the instant invention to produce CDMAB whose
4 cytotoxicity is mediated through ADCC.

5 It is yet an additional objective of the instant invention to produce CDMAB whose
6 cytotoxicity is mediated through CDC.

7 It is still a further objective of the instant invention to produce CDMAB whose
8 cytotoxicity is a function of their ability to catalyze hydrolysis of cellular chemical bonds.

9 A still further objective of the instant invention is to produce CDMAB which are
10 useful in a binding assay for diagnosis, prognosis, and monitoring of cancer.

11 Other objects and advantages of this invention will become apparent from the
12 following description wherein are set forth, by way of illustration and example, certain
13 embodiments of this invention.

14

15 Brief Description of the Figures:

16 The patent or application file contains at least one drawing executed in color. Copies of
17 this patent or patent application publication with color drawing(s) will be provided by the
18 Office upon request and payment of the necessary fee.

19 Figure 1. Representative micrographs showing the binding pattern obtained with H460-16-
20 2 (A) and the anti-CD44 (BU75) antibody (B) on tissues sections of tonsil from a normal
21 human tissue array. There is more intense and widely distributed staining of lymphocytes

1 with BU75 than with H460-16-2. The germinal center (green arrows) had weaker staining
2 for both antibodies. Magnification is 200X.

3 Figure 2. Representative micrograph of H460-16-2 binding to breast cancer tumor
4 (infiltrating duct carcinoma). The yellow and orange arrows in the panel point to stromal
5 cells and sheets of malignant cells respectively. Magnification is 100X.

6 Figure 3. Representative micrographs showing the binding pattern obtained with H460-16-
7 2 (A) and the anti-CD44 (BU75) antibody (B) on paget's disease breast tissue sections
8 from a human breast cancer tissue array. There is a membranous staining of malignant
9 cells with BU75 versus negative staining with H460-16-2. Magnification is 400X.

10 Figure 4. Representative micrographs showing the binding pattern obtained with H460-16-
11 2 (A) and the anti-Her2 (c-erbB-2) antibody (B) on medullary carcinoma from breast tissue
12 sections from a human breast cancer tissue array. There is strong membranous staining of
13 malignant cells with H460-16-2 versus negative staining with anti-Her2. Magnification is
14 200X.

15 Figure 5. Effect of H460-16-2, Cisplatin, H460-16-2 + Cisplatin or buffer control on tumor
16 growth in an established PC-3 prostate cancer model. The dashed line indicates the period
17 during which the antibody was administered. Data points represent the mean +/- SEM.

18 Figure 6. Effect of H460-16-2, Cisplatin, H460-16-2 + Cisplatin or buffer control on body
19 weight in an established PC-3 prostate cancer model.

20 Figure 7. Effect of H460-16-2, BU75 (positive control) or isotype control on MDA-MB-
21 231 breast cancer cell binding to hyaluronic acid (HA).

22 Figure 8. Effect of H460-16-2 or isotype control on cell cycle distribution of MDA-MB-
23 231 cells after treatment for 24 hrs.

24

1 Detailed Description Of The Invention:

2 Example 1

3 The hybridoma cell line H460-16-2 was deposited, in accordance with the
4 Budapest Treaty, with the American Type Culture Collection, 10801 University Blvd.,
5 Manassas, VA 20110-2209 on September 4, 2002, under Accession Number PTA-4621.
6 In accordance with 37 CFR 1.808, the depositors assure that all restrictions imposed on the
7 availability to the public of the deposited materials will be irrevocably removed upon the
8 granting of a patent.

9 **Antibody Production:**

10 H460-16-2 monoclonal antibody was produced by culturing the hybridoma in CL-
11 1000 flasks (BD Biosciences, Oakville, ON) with collections and reseeded occurring
12 twice/week. The antibody was purified according to standard antibody purification
13 procedures with Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Baie d'Urfé,
14 QC). It is within the scope of this invention to utilize monoclonal antibodies that are
15 human, humanized, chimerized or murine antibodies.

16

17 Example 2

18 **Normal Human Tissue Staining**

19 IHC studies were previously conducted to characterize H460-16-2 antigen
20 distribution in humans (S.N. 10/603,000) and in comparison to L178 (S.N. 10/647,818).
21 The current studies compare H460-16-2 to another antibody directed against CD44 (BU75)
22 since the H460-16-2 antigen may be a cancer variant of CD44 as determined previously by
23 biochemical methods. Binding of antibodies to 59 normal human tissues was performed
24 using a human normal organ tissue array (Imgenex, San Diego, CA). All primary
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1 antibodies (H460-16-2; BU75 anti-CD44 (BIOCAN Scientific Inc., Mississauga, ON); and
2 mouse IgG₁ negative control (Dako, Toronto, ON)) were diluted in antibody dilution
3 buffer (Dako, Toronto, ON) to a concentration of 5 µg/ml (found to be the optimal
4 concentration in previous optimization steps). The negative control antibody has been
5 shown to be negative to all mammalian tissues by the manufacturer. The procedure for
6 IHC is as follows.

7 Tissue sections were deparaffinized by drying in an oven at 58°C for 1 hr and
8 dewaxed by immersing in xylene 5 times for 4 min each in Coplin jars. Following
9 treatment through a series of graded ethanol washes (100%-75%) the sections were re-
10 hydrated in water. The slides were immersed in 10 mM citrate buffer at pH 6 (Dako,
11 Toronto, Ontario) then microwaved at high, medium, and low power settings for 5 min
12 each and finally immersed in cold PBS. Slides were then immersed in 3 percent hydrogen
13 peroxide solution for 6 min, washed with PBS 3 times for 5 min each, dried, incubated
14 with Universal blocking solution (Dako, Toronto, Ontario) for 5 min at room temperature.
15 H460-16-2, BU75 or isotype control antibody (directed towards *Aspergillus niger* glucose
16 oxidase, an enzyme which is neither present nor inducible in mammalian tissues) were
17 diluted in antibody dilution buffer (Dako, Toronto, Ontario) to its working concentration (5
18 µg/mL for each antibody) and incubated for 1 hr at room temperature. The slides were
19 washed with PBS 3 times for 5 min each. Immunoreactivity of the primary antibodies was
20 detected/visualized with HRP conjugated secondary antibodies as supplied (Dako Envision
21 System, Toronto, Ontario) for 30 min at room temperature. Following this step the slides
22 were washed with PBS 3 times for 5 min each and a color reaction developed by adding
23 DAB (3,3'-diaminobenzidine tetrahydrachloride, Dako, Toronto, Ontario) chromogen
24 substrate solution for immunoperoxidase staining for 10 min at room temperature.

1 Washing the slides in tap water terminated the chromogenic reaction. Following
2 counterstaining with Meyer's Hematoxylin (Sigma Diagnostics, Oakville, ON), the slides
3 were dehydrated with graded ethanols (75-100%) and cleared with xylene. Using mounting
4 media (Dako Faramount, Toronto, Ontario) the slides were coverslipped. Slides were
5 microscopically examined using an Axiovert 200 (Zeiss Canada, Toronto, ON) and digital
6 images acquired and stored using Northern Eclipse Imaging Software (Mississauga, ON).
7 Results were read, scored and interpreted by a pathologist.

8 Table 1 presents a summary of the results of H460-16-2 and BU75 anti-CD44
9 staining of an array of normal human tissues. The staining of tissues with H460-16-2 is
10 similar to that described previously (S.N. 10/603,000). It should be again noted that the
11 antigen is generally not present on cells in the vital organs, including the liver, kidney,
12 heart and lung. The H460-16-2 antibody does bind to macrophages and lymphocytes, and
13 their presence is observed in some of the organs in these sections. However, there was a
14 higher intensity of staining of lymphocytes seen with the BU75 anti-CD44 antibody
15 (Figure 1).

16 Tissues that were positive for H460-16-2 were also usually positive for BU75 anti-
17 CD44 (sometimes to a greater intensity). Tissues that were negative for H460-16-2 were
18 also generally negative for BU75 anti-CD44 albeit there are a few exceptions such as one
19 sample of esophagus and lymph node. These results demonstrate that H460-16-2 binds to a
20 smaller subset of the tissues recognized by the BU75 anti-CD44 antibody and within tissues
21 the intensity of staining is also sometimes less. These results show that the antigen for
22 H460-16-2 is not widely expressed on normal tissues, and that the antibody binds
23 specifically to a limited number of tissues in humans. It also supports the biochemical data

in that H460-16-2 is directed against an epitope of CD44, that is a different variant than the one recognized by the BU75 used for these IHC studies.

Table 1: Comparison of BU75 anti-CD44 and H460-16-2 IHC on Human Normal Tissue

Data Sheet		BU75		H460-16-2	
Sec. No.	Organ	Section Score	Tissue specificity	Section Score	Tissue specificity
1	Skin	+++	Keratinocytes of all layers except Stratum corneum	+++	Keratinocytes of all layers except Stratum corneum
2	Skin	+++	Keratinocytes of all layers except Stratum corneum	+++	Keratinocytes of all layers except Stratum corneum
3	Subcutis fat	-		-	
4	Breast	+	Myoepithelium	+	Myoepithelium
5	Breast	++	Ductular epithelium & Myoepithelium	+	Myoepithelium & Fibroblasts
6	Spleen	+++	Lymphocytes	++	Lymphocytes (more intense in the periaarteriolar area)
7	Spleen	+++	Lymphocytes (more intense in the periaarteriolar area)	+++	Lymphocytes (more intense in the periaarteriolar area)
8	Lymph node	+++	Lymphocytes	+	Lymphocytes
9	Lymph node	+	Lymphocytes	-	
10	Skeletal muscle	+/-	Blood vessels	+/-	Blood vessels
11	Nasal Mucosa	+++	Mucosal epithelium (basal layers)	CD	
12	Lung	++	SMF & Macrophages	++	Lymphocytes & Macrophages
13	Lung	++	Alveolar epithelium & Macrophages	+++	Lymphocytes & Macrophages
14	Bronchus	+++	Chondrocytes	NR	
15	Heart	-		-	
16	Salivary gland	+++	Ductular & acinar epithelium	++	Ductular & acinar epithelium
17	Liver	+++	Kupffer cells	+++	Kupffer cells
18	Liver	+++	Kupffer cells	+++	Kupffer cells
19	Liver	-		-	
20	Gall bladder	+++	Mucosal basal epithelium & Lymphocytes	+	Mucosal basal epithelium & Lymphocytes
21	Pancreas	++	Acinar epithelium	+	Acinar epithelium
22	Pancreas	+++	Acinar epithelium	++	Acinar epithelium
23	Tonsil	+++	Lymphocytes (less intense at germinal center)	++	Lymphocytes (less intense at germinal center)
24	Esophagus	++	Mucosal basal epithelium layers & Lymphocytes	-	
25	Esophagus	+++	Basal mucosal epithelial layers & Lymphocytes	+++	Basal mucosal epithelial layers
26	Stomach body	+++	Glandular epithelium in the basal glands & Lymphocytes	++	Glandular epithelium in the basal glands & Lymphocytes
27	Stomach body	+++	Glandular epithelium in the basal glands & Lymphocytes	+++	Glandular epithelium in the basal glands & Lymphocytes
28	Stomach antrum	+++	Glandular epithelium in the basal glands & Lymphocytes	+++	Glandular epithelium in the basal glands & Lymphocytes
29	Stomach smooth muscle	++	Blood vessels & Peripheral nerve fibers	++	Blood vessels & Fibroblasts
30	Duodenum	+++	Lymphocytes in lamina propria	++	Lymphocytes in lamina propria
31	Small bowel	++	Glandular epithelium & Lymphocytes	+	Lymphocytes in lamina propria
32	Small bowel	+++	Glandular epithelium in the basal glands & Lymphocytes in Lymphoid follicles (Less intense in the germinal center)	+++	Lymphocytes in Lymphoid follicles (Less intense in the germinal center)
33	Appendix	+++	Glandular epithelium in the basal glands & Lymphocytes in Lymphoid follicles (Less intense in the germinal center)	+++	Lymphocytes in lamina propria +/- Glandular epithelium of basal glands
34	Colon	+++	Glandular epithelium in the basal glands & Lymphocytes in Lymphoid follicles (Less intense in the germinal center)	+++	Lymphocytes & peripheral nerve fibers
35	Colon	++	Lymphocytes & endothelium	+++	Lymphocytes in lamina propria
36	Rectum	++	Lymphocytes & Endothelium	+++	Lymphocytes & Fibroblasts
37	Kidney cortex	++	Endothelium of blood vessels	+/-	Interstitial blood vessels
38	Kidney cortex	+/-	Tubular epithelium	+/-	Tubular epithelium
39	Kidney Medulla	++	Endothelium of blood vessels	+/-	SMF & Fibroblasts
40	Urinary Bladder	++	Transitional epithelium & endothelium of blood vessels	++	Lymphocytes & Macrophages +/- Transitional epithelium & endothelium of blood vessels
41	Prostate	+++	Myoepithelium	+++	Myoepithelium
42	Prostate	+++	Myoepithelium	++	Myoepithelium
43	Seminal Vesicle	+/-	Endothelium & SMF	+/-	Mucosal epithelium, Endothelium & SMF
44	Testis	+/-	Endothelium of blood vessels	+/-	Endothelium of blood vessels & fibroblasts
45	Endometrium proliferative	+++	Stroma & endothelium of blood vessels & Adjacent myometrium	++	Stroma & endothelium of blood vessels & Adjacent myometrium
46	Endometrium secretory	+++	Glandular epithelium, Stroma & Endothelium	++	Glandular epithelium, Stroma & Endothelium
47	Myometrium	+++	SMF	+++	SMF
48	Uterine cervix	+++	Basal layers of mucosal epithelium & Endothelium of blood vessels	+++	Basal layers of mucosal epithelium
49	Salpinx	++	SMF & endothelium of blood vessels	+	SMF & Fibroblasts
50	Ovary	++	Endothelium & SMF of blood vessels	+/-	SMF of blood vessels
51	Placenta villi	+/-	Endothelium of blood vessels	+/-	Endothelium of blood vessels
52	Placenta villi	+/-	Endothelium of blood vessels	+/-	Endothelium of blood vessels
53	Umbilical cord	-		-	
54	Adrenal gland	+/-	Endothelium of blood vessels	+/-	Endocrine glands
55	Thyroid	+/-	Endothelium of blood vessels	+/-	Parafollicular cells & Endothelium of blood vessels
56	Thymus	++	Lymphocytes	+/-	Lymphocytes
57	Brain white matter	-		-	
58	Brain gray matter	-		-	
59	Cerebellum	-		-	

Abbreviations: SMF: Smooth muscle fibers, NR: The section is not representative.

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1 Example 3

2 **Human Breast Tumor Tissue Staining**

3 Previous IHC studies were undertaken to determine the cancer association of the
4 H460-16-2 antigen with human breast cancers and whether the H460-16-2 antibody was
5 likely to recognize human cancers (S.N. 10/603,000) and how it compared to anti-CD44
6 staining with L178 (S.N. 10/647,818). Currently, a comparison was made for BU75 anti-
7 CD44 staining, c-erbB-2 anti-Her2 and an antibody directed towards *Aspergillus niger*
8 glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissues
9 (negative control). A breast cancer tissue array derived from 50 breast cancer patients and
10 10 samples derived from non-neoplastic breast tissue in breast cancer patients was used
11 (Imgenex Corporation, San Diego, CA). The following information was provided for each
12 patient: age, sex, American Joint Committee on Cancer (AJCC) tumor stage, lymph node,
13 estrogen receptor (ER) and progesterone receptor (PR) status. The procedure for IHC from
14 Example 5 was followed. All antibodies were used at a working concentration of 5 µg/mL
15 except for anti-Her2 which was used at a concentration of 1.5 µg/mL.

16 Tables 2 and 3 provide summaries of H460-16-2 and BU75 anti-CD44 antibody
17 staining of breast cancer tissue arrays respectively. Each array contained tumor samples
18 from 50 individual patients. Overall, 62 percent of the 50 patients tested were positive for
19 H460-16-2 antigen compared to 73 percent for BU75 anti-CD44. In cases where both
20 H460-16-2 and BU75 anti-CD44 stained the same tissue, 45 percent of the samples had
21 higher intensity staining with the BU75 anti-CD44 in comparison to H460-16-2. For the
22 H460-16-2 and BU75 antigen, 4 and 8 out of 10 normal breast tissue samples from breast
23 cancer patients were positive, respectively. No clear correlation between estrogen and

progesterone receptor status was evident. It also did not appear to be a trend to greater positive expression of the H460-16-2 and CD44 antigen with higher tumor stage.

Table 2: Human Breast Tumor IHC Summary for H460-16-2

			Binding Score					Total Positive	% Positive
		Total #	-	+/-	+	++	+++		
Patient Samples	Tumor	50	19	19	4	3	5	31	62
	Normal	10	0	1	0	2	1	4	40
ER Status	ER+	28	13	13	1	1	0	15	54
	ER-	22	6	8	3	0	5	16	73
	Unknown	0	0	0	0	0	0	0	0
PR Status	PR+	19	9	8	1	1	0	10	53
	PR-	30	8	14	3	0	5	22	73
	Unknown	1	0	1	0	0	0	1	100
AJCCTumorStage	T1	4	2	1	1	0	0	2	50
	T2	21	6	9	1	1	4	15	71
	T3	20	9	9	1	0	1	11	55
	T4	5	2	2	1	0	0	3	60

Table 3: Human Breast Tumor IHC Summary for Anti-CD44 (BU75)

			Binding Score					Total Positive	% Positive
		Total #	-	+/-	+	++	+++		
Patient Samples	Tumor	48	13	6	13	7	9	35	73
	Normal	10	2	0	3	2	3	8	80
ER Status	ER+	27	8	4	10	2	3	19	70
	ER-	21	5	2	3	5	6	16	76
	Unknown	0	0	0	0	0	0	0	0
PR Status	PR+	18	4	2	8	2	2	14	78
	PR-	29	8	4	5	5	7	21	72
	Unknown	1	1	0	0	0	0	0	0
AJCC Tumor Stage	T1	4	1	2	0	1	0	3	75
	T2	20	7	0	6	2	5	13	65
	T3	19	5	2	5	3	4	14	74
	T4	5	0	2	2	1	0	5	100

The H460-16-2 staining was specific for cancerous cells in comparison to normal cells as demonstrated in Figure 2 where stromal cells were clearly negative and sheets of malignant cells were highly positive. The cellular localization pattern seen with the H460-16-2 antigen was confined to the cell membrane in the majority of cases. The BU75 CD44 antibody stained more breast cancer samples and showed a higher degree of membrane than cytoplasmic localization compared to H460-16-2 (Table 4). BU75 anti-CD44 also stained malignant cells of Paget's disease, which was not the case for H460-16-2 (Figure

1 3). The samples of normal tissue from breast cancer patients that were positive for H460-
2 16-2 staining were also positive for BU75 anti-CD44 staining.

3 In comparison to c-erbB-2, H460-16-2 showed a completely different staining
4 profile where 16 out of the 31 breast tumor tissue samples that were positive for the H460-
5 16-2 antigen were negative for Her2 expression indicating a yet unmet targeted therapeutic
6 need for breast cancer patients (Table 5, Figure 4). There were also differences in the
7 intensity of staining between the breast tumor tissue sections that were positive for both
8 H460-16-2 and Her2; some breast tumor tissue sections that were highly positive for the
9 H460-16-2 antigen were only mildly positive for Her2 and vice versa again illustrating that
10 H460-16-2 would therapeutically target a different cohort of breast cancer patients. The c-
11 erbB-2 antibody also positively stained one of the normal breast tissue sections.

12 These results suggest the antigen for H460-16-2 may be expressed by almost two
13 thirds of breast cancer patients. In addition, the majority of those suitable for H460-16-2
14 treatment would not have been suitable for anti-Her2 treatment. The staining pattern
15 showed that in patient samples, the antibody is highly specific for malignant cells and the
16 H460-16-2 antigen is localized to the cell membrane thereby making it an attractive
17 drugable target. The similar albeit more limited staining of H460-16-2 versus BU75 anti-
18 CD44 antibody again demonstrates the likelihood of the H460-16-2 epitope being a more
19 restricted variant of CD44.

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3 Table 4: Comparison of BU75 anti-CD44 and H460-16-2 IHC on Human Tumor and Normal Breast Tissue

Data sheet				BU75		H460-16-2	
Sec. No.	Sex	Age	Diagnosis	Section Score	Tissue specificity	Section Score	Tissue specificity
1	F	28	Infiltrating duct carcinoma	+/-	Tumor cells & Stroma	+/-	Tumor cells
2	F	71	Solid papillary carcinoma	+	Tumor cells & Stroma	+	Tumor cells
3	F	26	Infiltrating duct carcinoma	+/-	Tumor cells & Stroma	-	
4	F	43	Infiltrating duct carcinoma	+	Tumor cells	-	
5	F	39	Infiltrating duct carcinoma	+++	Tumor cells	+	Tumor cells & Necrotic area
6	F	46	Ductal carcinoma in situ	-		-	
7	F	47	Infiltrating duct carcinoma	+++	Tumor cells	+++	Tumor cells
8	M	67	Infiltrating duct carcinoma	+	Tumor cells & Stroma	-	
9	F	33	Infiltrating duct carcinoma	+++	Tumor cells	-	Tumor cells & Stroma
10	F	47	Infiltrating duct carcinoma	+	Tumor cells & Stroma	+	Tumor cells & Stroma
11	F	49	Invasive Lobular carcinoma	-		-	
12	F	46	Infiltrating duct carcinoma	+	Tumor cells & Stroma	+/-	Tumor cells & Stroma
13	F	39	Infiltrating duct carcinoma	-		+/-	
14	F	43	Infiltrating lobular carcinoma	+	Tumor cells	+	Tumor cells
15	F	54	Infiltrating lobular carcinoma	+++	Tumor cells	+/-	Tumor cells
16	F	58	Infiltrating duct carcinoma	++	Tumor cells & Stroma	++	Tumor cells
17	F	37	Infiltrating duct carcinoma	-	Tumor's cells +/- Stroma	-	Tumor's cells +/- Stroma
18	F	43	Infiltrating duct carcinoma	+++	Tumor cells	+	Tumor cells +++ Stroma
19	F	51	Infiltrating duct carcinoma	+++	Tumor cells	++	Tumor cells
20	F	80	Medullary carcinoma	+++	Tumor cells & Lymphocytes	+++	Tumor cells
21	F	36	Infiltrating duct carcinoma	NR		+++	Tumor cells
22	F	59	Infiltrating duct carcinoma	+	Tumor cells	+/-	Tumor cells ++ Stroma
23	F	34	Ductal carcinoma in situ	+	Tumor cells	+/-	Tumor cells & Necrotic area
24	F	54	Infiltrating duct carcinoma	-	Tumor cells & Stroma	+/-	Tumor cells
25	F	47	Infiltrating duct carcinoma	++	Tumor cells	+	Tumor cells
26	F	53	Infiltrating duct carcinoma	+	Tumor cells & Lymphocytes	+/-	Tumor cells ++ Stroma
27	F	59	Infiltrating duct carcinoma	+	Tumor cells ++ Stroma	+/-	Tumor cells +++ Lymphocytes
28	F	60	Sigmet ring cell carcinoma	F		-	
29	F	37	Infiltrating duct carcinoma	+/-	Tumor cells & Stroma	+/-	Tumor cells
30	F	46	Infiltrating duct carcinoma	-	Tumor cells + Stroma	+/-	Tumor cells & Stroma
31	F	35	Infiltrating duct carcinoma	-		-	
32	F	47	Infiltrating duct carcinoma	++	Tumor cells	-	Tumor cells +/- Necrotic area
33	F	54	Infiltrating duct carcinoma	+	Tumor cells	-	
34	F	47	Infiltrating duct carcinoma	+++	Tumor cells	+++	Tumor cells
35	F	41	Infiltrating duct carcinoma	-		-	
36	F	38	Infiltrating duct carcinoma	++	Tumor cells	+	Tumor cells
37	F	55	Infiltrating duct carcinoma	-	Tumor cells ++ Stroma	-	
38	F	65	Infiltrating duct carcinoma	-	Tumor cells ++ Stroma	-	Tumor cells +/- Stroma
39	M	66	Infiltrating duct carcinoma	-	Tumor cells & Necrotic area	-	
40	F	44	Infiltrating duct carcinoma	+/-	Tumor cells & Stroma	-	Tumor cells + Infiltrating Lymphocytes
41	F	52	Metastatic carcinoma in Lymph node	++	Tumor cells & Stroma	+/-	Tumor cells & Stroma
42	F	32	Metastatic carcinoma in Lymph node	+	Tumor cells	-	
43	F	58	Metastatic carcinoma in Lymph node	++	Tumor cells	++	Tumor cells
44	F	52	Metastatic carcinoma in Lymph node	-		-	
45	F	58	Metastatic carcinoma in Lymph node	-	Tumor cells +++ Lymphocytes	+/-	Tumor cells & Lymphocytes
46	F	38	Metastatic carcinoma in Lymph node	+/-	Tumor cells & Lymphocytes	-	Tumor cells + Lymphocytes
47	F	45	Metastatic carcinoma in Lymph node	++	Tumor cells	+	Tumor cells
48	F	45	Metastatic carcinoma in Lymph node	+	Tumor cells	+/-	Tumor cells
49	F	29	Metastatic carcinoma in Lymph node	+++	Tumor cells	+++	Tumor cells
50	F	61	Metastatic carcinoma in Lymph node	+/-	Tumor cells +++ Lymphocytes	+/-	Tumor cells ++ Lymphocytes
51	F	46	Nipple	+++	Keratinocytes (all layers except Stratum corneum)	++	Keratinocytes (all layers except Stratum corneum)
52	F	47	Nipple	+	Tumor cells	-	
53	F	40	Normal Breast	++	Ductular epithelium	-	
54	F	43	Normal Breast	+++	Ductular epithelium & Myoepithelium	+++	Myoepithelium
55	F	40	Normal Breast	++	Ductular epithelium & Myoepithelium	++	Myoepithelium
56	F	40	Normal Breast	+++	Myoepithelium +/- Ductular epithelium	+/-	Myoepithelium & Fibroblasts
57	F	45	Normal Breast	-		-	
58	F	44	Normal Breast	-		-	
59	F	37	Normal Breast	+	Ductular basement membrane +/- Ductular epithelium	-	
60	F	51	Normal Breast	+	Myoepithelium & Endothelium	-(PD)	

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3 Table 5: Comparison of c-erbB-2 anti-Her2 and H460-16-2 IHC on Human Tumor and Normal Breast Tissue

Data sheet				c-erbB-2		H460-16-2	
Sec. No.	Sex	Age	Diagnosis	Section Score	Tissue specificity	Section Score	Tissue specificity
1	F	28	Infiltrating duct carcinoma	+	Tumor cells	+/-	Tumor cells
2	F	71	Solid papillary carcinoma	-		+	Tumor cells
3	F	26	Infiltrating duct carcinoma	+/-	Tumor cells	-	
4	F	43	Infiltrating duct carcinoma	+/-	Tumor cells	-	
5	F	39	Infiltrating duct carcinoma	NR		+	Tumor & Necrotic area
6	F	46	Ductal carcinoma in situ	-		-	
7	F	47	Infiltrating duct carcinoma	+++	Tumor cells	+++	Tumor cells
8	M	67	Infiltrating duct carcinoma	-		-	
9	F	33	Infiltrating duct carcinoma	+++	Tumor cells	-	Tumor cells
10	F	47	Infiltrating duct carcinoma	++	Tumor cells	+	++ Stroma
11	F	49	Invasive Lobular carcinoma	PD		-	Tumor cells & Stroma
12	F	46	Infiltrating duct carcinoma	-		+/-	
13	F	39	Infiltrating duct carcinoma	+++	Tumor cells	-	Tumor cells & Stroma
14	F	43	Infiltrating lobular carcinoma	-		+	Tumor cells
15	F	54	Infiltrating lobular carcinoma	-		+/-	Tumor cells
16	F	58	Infiltrating duct carcinoma	-		+	Tumor cells
17	F	37	Infiltrating duct carcinoma	+++	Tumor cells	-	++ Necrotic area
18	F	43	Infiltrating duct carcinoma	-		+	Tumor cells
19	F	51	Infiltrating duct carcinoma	+	Tumor cells	++	+++ Stroma
20	F	80	Medullary carcinoma	-		+++	Tumor cells
21	F	36	Infiltrating duct carcinoma	NR		+++	Tumor cells & Stroma
22	F	59	Infiltrating duct carcinoma	-		+/-	Tumor cells
23	F	34	Ductal carcinoma in situ	+++	Tumor cells	+/-	Tumor's cells & Necrotic area
24	F	54	Infiltrating duct carcinoma	+	Tumor cells	+/-	Tumor cells
25	F	47	Infiltrating duct carcinoma	-		+	Tumor cells
26	F	53	Infiltrating duct carcinoma	+++	Tumor cells	+/-	Tumor cells
27	F	59	Infiltrating duct carcinoma	+	Tumor cells	+/-	++ Stroma
28	F	60	Signet ring cell carcinoma	-		-	Tumor cells
29	F	37	Infiltrating duct carcinoma	+++	Tumor cells	+/-	+++ Lymphocytes
30	F	46	Infiltrating duct carcinoma	-		+/-	Tumor cells
31	F	35	Infiltrating duct carcinoma	-		-	Tumor cells & Stroma
32	F	47	Infiltrating duct carcinoma	+++	Tumor cells	-	Tumor cells
33	F	54	Infiltrating duct carcinoma	-		-	+/- Necrotic area
34	F	47	Infiltrating duct carcinoma	+++	Tumor cells	+++	Tumor cells
35	F	41	Infiltrating duct carcinoma	-		-	
36	F	38	Infiltrating duct carcinoma	++	Tumor cells	+	Tumor cells
37	F	55	Infiltrating duct carcinoma	+/-	Tumor cells	-	
38	F	65	Infiltrating duct carcinoma	-		-	Tumor cells
39	M	66	Infiltrating duct carcinoma	-		-	+/- Stroma
40	F	44	Infiltrating duct carcinoma	-		-	
41	F	52	Metastatic carcinoma in Lymph node	-		+/-	Tumor cells
42	F	32	Metastatic carcinoma in Lymph node	-		-	+ Infiltrating Lymphocytes
43	F	58	Metastatic carcinoma in Lymph node	++	Tumor cells	+	Tumor cells & Stroma
44	F	52	Metastatic carcinoma in Lymph node	+++	Tumor cells	-	
45	F	58	Metastatic carcinoma in Lymph node	-		+/-	Tumor cells & Lymphocytes
46	F	38	Metastatic carcinoma in Lymph node	++	Tumor cells	-	Tumor cells
47	F	45	Metastatic carcinoma in Lymph node	-		+	+ Lymphocytes
48	F	45	Metastatic carcinoma in Lymph node	-		+/-	Tumor cells
49	F	29	Metastatic carcinoma in Lymph node	-		+++	Tumor cells
50	F	61	Metastatic carcinoma in Lymph node	-		+/-	Tumor cells
51	F	46	Nipple	-		++	++ Lymphocytes
52	F	47	Nipple	+++	Tumor cells	-	Keratinocytes
53	F	40	Normal Breast	-		-	(all layers except Stratum corneum)
54	F	43	Normal Breast	-		+++	
55	F	40	Normal Breast	-	Ductular epithelium	++	Myoepithelium
56	F	40	Normal Breast	-		+/-	Myoepithelium
57	F	45	Normal Breast	-		-	Myoepithelium & Fibroblasts
58	F	44	Normal Breast	-		-	
59	F	37	Normal Breast	-		-	
60	F	51	Normal Breast	-		- (PD)	

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1 Example 4

2 ***In Vivo* PC-3 Established Chemotherapy Combination Tumor Experiments**

3 With reference to Figures 5 and 6, 6 to 8 week old male SCID mice were implanted
4 with 1 million PC-3 human prostate cancer cells in 100 microlitres saline injected
5 subcutaneously in the scruff of the neck. Tumor growth was measured with calipers every
6 week. When the majority of the cohort reached a tumor volume of 80 mm³ (range 50-130
7 mm³) at 21 days post-implantation, 8 mice were randomly assigned into each of 4
8 treatment groups. H460-16-2 antibody, the chemotherapeutic drug Cisplatin, the
9 combination of H460-16-2 and Cisplatin or buffer control was administered
10 intraperitoneally with 15 or 6 mg/kg of antibody or Cisplatin respectively at a volume of
11 300 microliters after dilution from the stock concentration with a diluent that contained 2.7
12 mM KCl, 1 mM KH₂PO₄, 137 mM NaCl and 20 mM Na₂HPO₄. H460-16-2 or buffer
13 control was then administered 4 times per week for the first week followed by 3 times per
14 week for 11 doses in total in the same fashion until day 41 post-implantation. Cisplatin
15 was administered on days 0, 5, 10 and 15 of the antibody treatment period. Tumor growth
16 was measured about every seventh day with calipers until day 48 post-implantation or until
17 individual animals reached the CCAC end-points. Body weights of the animals were
18 recorded for the duration of the study. At the end of the study all animals were euthanised
19 according to CCAC guidelines.

20 Using a paired t-test, there was a post-treatment tumor burden reduction (Figure 5)
21 associated with treatment with either Cisplatin or the combination of H460-16-2 and
22 Cisplatin. At day 48 (7 days post-treatment) Cisplatin and H460-16-2 treatment was
23 significantly more effective in suppressing tumor growth shortly after the treatment period
24 than buffer control (p<0.0001), Cisplatin treatment alone (p=0.004) or H460-16-2

1 treatment alone ($p < 0.0001$). PC-3 is a cachexic model of prostate cancer, in which
2 increased tumor burden and disease progression in the xenograft model is accompanied by
3 weight loss. As demonstrated by the mean weights shown in Figure 6, the mice in all the
4 groups experienced severe weight loss. In this study, mice in all groups showed a weight
5 loss of approximately 23 to 35 percent by the end of the treatment period. The group
6 treated with H460-16-2 showed the smallest degree of weight loss (21.7 percent). Shortly
7 after the end of treatment, there was no additional significant loss of body weight
8 associated with H460-16-2 plus Cisplatin treatment in comparison to the buffer control
9 ($p = 0.5042$). Therefore H460-16-2 plus Cisplatin lowered the tumor burden in comparison
10 to a buffer control in a well-recognized model of human breast cancer disease. These
11 results suggest pharmacologic and pharmaceutical benefits of this antibody for cancer
12 therapy in mammals, including man.

13 Example 5

14 **Hyaluronic Acid (HA) Binding Assay**

15 MDA-MB-231 cells (shown previously to express the H460-16-2 antigen (CD44)
16 by FACS analysis) were dissociated after aspirating the spent media from the tissue culture
17 plates, washing the plates with PBS, adding 5 mL of dissociation buffer to each plate and
18 incubating the plates at 37°C until the cells detached. Cells were then counted and
19 collected into 50 mL tube(s). Cells were spun at 1200 rpm for 5 min and resuspended in
20 media to yield 1-5 million cells/mL. One mL was then added to each well of a 2 mL deep
21 well. Cells were pelleted by spinning the plate at 1200 rpm for 5 min and excess
22 supernatant was removed by inverting the plate onto paper towels. Deep well plates were
23 then gently vortexed to dislodge and break up the cell pellets. One mL of H460-16-2,
24 BU75 (positive control, BIOCAN Scientific Inc., Mississauga, ON) or isotype negative

1 control (107.3, BD Biosciences, Oakville, ON) antibody was added to each well and then
2 mixed by gentle vortexing. Plates were then incubated at 37°C for 2 hrs. Meanwhile, 48-
3 well plates were coated with HA by incubating 300 µl of 4 mg/mL HA stock solution/well
4 for 1-2 hrs at 37°C. After incubation, excess HA was aspirated off and the plates(s) were
5 allowed to completely air dry in the laminar flow hood. After antibody-cell incubation was
6 completed, the cells were again pelleted at 1200 rpm for 5 min. Supernatant was removed
7 by inverting the deep well on paper towels. The deep well was again vortexed to dissociate
8 the cell pellets followed by addition of 1.2 mL of 2 µM calcein-am, in PBS containing
9 MgCl₂ and CaCl₂, to each well. Cells were resuspended and 250 µl/well was transferred to
10 the HA coated plate. HA coated plates were then incubated at 37°C for 2 hrs to allow
11 adhesion. After incubation, unattached cells were removed through aspiration. Each well
12 was then washed 2-3 times with PBS containing MgCl₂ and CaCl₂ in order to remove any
13 unattached cells or cell clumps. The plates were read in a Perkin-Elmer HTS7000
14 fluorescence plate reader and the data was analyzed in Microsoft Excel and the results
15 were tabulated in Table 6 or Figure 7. The results from an average of 6 separate
16 experiments revealed that an average of 1.87 (+/- 1.01) µg/mL of H460-16-2 was required
17 in order to cause a 50 percent reduction in the binding of MDA-MB-231 cells to HA
18 (Table 6). The effect of H460-16-2 on MDA-MB-231 cell binding to HA was dose
19 dependent; 20 µg/mL of H460-16-2 resulted in over a 60 percent reduction in cell binding
20 to HA (Figure 7). These results indicated that H460-16-2 interacts with, at least in part, the
21 region(s) on CD44 that are responsible for binding to HA and consequently could be
22 elucidating its anti-cancer effects through down regulation of angiogenesis or tumor
23 invasiveness through the ECM.

24

1 Table 6: Summary of Effect of H460-16-2 on MDA-MB-231 Cell Binding to HA

Experiment	Concentration of Antibody to Yield 50 Percent Adhesion ($\mu\text{g/mL}$)
1	2.42
2	0.99
3	2.56
4	0.70
5	1.87
6	1.06
Average	1.87
Standard Deviation	1.01

2

3 Example 6

4 **Cell Cycling Assay**

5 The effect of H460-16-2 on the cell cycle of MDA-MB-231 breast cancer cells was
6 evaluated using FACS analysis. H460-16-2 antibody (0, 0.2, 2.0 and 20 $\mu\text{g/mL}$) or isotype
7 control (clone 107.3, BD Biosciences, Oakville, ON) was incubated with MDA-MB-231
8 breast cancer cells for 24, 48 and 72 hrs. Treated and untreated cells were stained with
9 propidium iodide and single cells were analyzed by flow cytometry to assess relative DNA
10 content. The acquired data set was analyzed using BD CellQuest, by gating on the single
11 cells population as well as cells showing hypo-diploid staining. After this analysis, cells
12 treated with H460-16-2 for 24 hrs showed an overall decrease in the percentage of cycling
13 cells as well as a dose dependent increase in the sub- G_1 population. Cells that appeared in
14 the sub- G_1 population are cells that have lost DNA due to the loss of cell membrane
15 integrity and may represent the apoptotic cell population (Figure 8). This data
16 demonstrated that H460-16-2 had an effect on MDA-MB-231 cell cycling and this effect
17 led to a dose dependent increase in the number of apoptotic cells.

18 The preponderance of evidence shows that H460-16-2 mediates anti-cancer effects
19 through ligation of a carbohydrate dependent conformational epitope present on a variant
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1 of CD44 and that this epitope is at least partially involved in the binding of CD44 to HA.
2 There is also evidence that binding of H460-16-2 to this epitope can lead to apoptosis on
3 the corresponding cell. It has been shown, in S.N. 10/713,451, H460-16-2 antibody can be
4 used to immunoprecipitate the cognate antigen from expressing cells such as MDA-MB-
5 231 cells. Further it could be shown that the H460-16-2 antibody could be used in
6 detection of cells and/or tissues which express a CD44 antigenic moiety which specifically
7 binds thereto, utilizing techniques illustrated by, but not limited to FACS, cell ELISA or
8 IHC.

9 Thus, it could be shown that the immunoprecipitated H460-16-2 antigen can inhibit
10 the binding of H460-16-2 to such cells or tissues using such FACS, cell ELISA or IHC
11 assays. Further, as with the H460-16-2 antibody, other anti-CD44 antibodies could be used
12 to immunoprecipitate and isolate other forms of CD44 antigen, and the antigen can also be
13 used to inhibit the binding of those antibodies to the cells or tissues that express the antigen
14 using the same types of assays. It could also be shown that if an anti-CD44 antibody that
15 recognizes all forms of CD44 (i.e. a pan-CD44 antibody) were used to isolate its cognate
16 antigen, then that antigen could also inhibit the binding of H460-16-2 antigen to cells or
17 tissues that express that antigen, thus also demonstrating the binding of H460-16-2 to an
18 epitope of CD44 on cells and tissues expressing that antigen. Alternatively, a comparison
19 of H460-16-2 and pan-CD44 antibody in assays such as competitive binding assays.
20 ELISA, cell ELISA, FACS or the like, where both antibodies are present can also
21 demonstrate the binding of H460-16-2 to an epitope of CD44 on cells and tissues
22 expressing that antigen.

23 All patents and publications mentioned in this specification are indicative of the
24 levels of those skilled in the art to which the invention pertains. All patents and

1 publications are herein incorporated by reference to the same extent as if each individual
2 publication was specifically and individually indicated to be incorporated by reference.

3 It is to be understood that while a certain form of the invention is illustrated, it is
4 not to be limited to the specific form or arrangement of parts herein described and shown.

5 It will be apparent to those skilled in the art that various changes may be made without
6 departing from the scope of the invention and the invention is not to be considered limited
7 to what is shown and described in the specification. One skilled in the art will readily
8 appreciate that the present invention is well adapted to carry out the objects and obtain the
9 ends and advantages mentioned, as well as those inherent therein. Any oligonucleotides,
10 peptides, polypeptides, biologically related compounds, methods, procedures and
11 techniques described herein are presently representative of the preferred embodiments, are
12 intended to be exemplary and are not intended as limitations on the scope. Changes therein
13 and other uses will occur to those skilled in the art which are encompassed within the spirit
14 of the invention and are defined by the scope of the appended claims. Although the
15 invention has been described in connection with specific preferred embodiments, it should
16 be understood that the invention as claimed should not be unduly limited to such specific
17 embodiments. Indeed, various modifications of the described modes for carrying out the
18 invention which are obvious to those skilled in the art are intended to be within the scope
19 of the following claims.